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Dopamine Transport into a Single Cell in a Picoliter Vial

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The analysis of chemical events in small volumes requires careful manipulation of samples and sensitive detection methods. Here, we describe the measurement of the neurotransmitter dopamine in a picoliter vial with electrochemical techniques. The vials were fabricated from fused-silica capillaries that provided a transparent container suitable for the observation and manipulation of a biological cell, sample solutions, and electrodes. Evaporation of the sample was prevented with a mineral oil layer, allowing for experiments lasting for several minutes. The small volume of these vials (100−200 pL) allows rapid mixing of all of the solution reagents. Similarly, the small volume allows exhaustive electrolysis of the vial contents with a 3-μm radius, disk-shaped carbon fiber microelectrode within 60 s. Fast-scan cyclic voltammetry at carbon fiber microelectrodes was used to monitor the concentration of analyte in the vial without depleting its contents. The concentration of dopamine introduced by pneumatic injection remained stable when sampled by cyclic voltammetry, and no evidence for adsorption to the walls was observed. However, when the vial contained a single HEK-293 cell transfected to express the dopamine transporter, the dopamine concentration decreased with time at a rate consistent with the uptake kinetics mediated by the transporter located on the cell membrane.

To detect small amounts of a chemical compound, miniaturized analytical techniques are advantageous. For example, separation techniques such as microbore liquid chromatography[1] and capillary electrophoresis[2] with electrochemical detection allow for analysis of ultrasmall injection volumes. This facilitates trace detection because it minimizes the need for sample dilution. However, to characterize dynamic chemical events in small volumes with minimal dilution, handling techniques for nano- and picoliter volumes are required.

Small-volume techniques are particularly useful for the investigation of the chemical contents of single biological cells. Such investigations can reveal unique distributions that are masked when a population of cells is examined. For example, individual vesicles from *Aplysia californica* were shown to contain only taurine, whereas previous studies on populations of cells had suggested a uniform content of it and another unidentified compound.[3] Recent advances in low-volume handling techniques for identification of chemical content in individual cells include NMR in picoliter volumes[4] and capillary electrophoresis with MALDI-MS detection for the analysis of single cells in nanoliter volume vials.[5]

To study chemical dynamics in small volumes, small containers with well-defined volumes are required along with chemical detectors suitable for the detection of small numbers of molecules. In this way, chemical events can be monitored that would be impossible to view in larger volumes because of analyte dilution or diffusional dispersion. Confinement of solution volumes has been accomplished in a variety of ways. One approach is to use aqueous droplets of picoliter volume in immiscible organic media for the diffusional delivery of reagents.[6] Nanoliter volume microvials have been machined in silicon for the introduction of samples into a capillary electrophoretic column.[7] Smaller, transparent vials (0.7 to 400 pL) have been fabricated in polystyrene.[8] Laser ablation of glass microscope coverslips has been used to fabricate 350 pL vials.[9] The coupling of optical fiber bundle etching with metal deposition techniques has recently resulted in optoelectrochemical microring arrays, allowing for simultaneous electrochemical sensing and imaging within microenvironments.[10]

The coupling of small volume handling techniques and electrochemical detection allows for the study of dynamic events at a single cell. Events of interest include the release or uptake of minute amounts of analyte, and these are quickly diluted in bulk solution. Electrochemical sensors have been fabricated in the base of a 600 pL well,[11] and have been used to measure release of lactate from a single heart cell in response to various chemical stressors.[12] Carbon-fiber microelectrodes, sensors with dimensions in the

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micrometer range, microsecond temporal response, and excellent sensitivity to electroactive species, have been used to characterize release of neurohormones from single cells by confining the measurement volume to a thin layer between the cell and the electrode.[13]

In this study, we report the development of a technique to measure the active transport of electroactive neurotransmitters into a single cell in real time. To achieve this, a transparent vial with a volume of 100−200 pL was fabricated from a fused-silica capillary. The vial was characterized with both amperometry and fast-scan cyclic voltammetry at carbon-fiber microelectrodes. The small dimensions of the vial allow for the measurement of total analyte concentration without losses from diffusion or dilution. To illustrate the utility of this approach, transport of dopamine into a cell expressing the human dopamine transporter was examined. This transporter is the major contributor to the regulation of the neurotransmitter dopamine in the extracellular space of the brain,[14] and thus, its rate and mechanism of action are of considerable interest. The uptake rates compare well with literature reports of uptake into populations of cells determined with the rotating disk electrode.[15]

EXPERIMENTAL SECTION

Vial Fabrication. Fifteen different vials of picoliter volume were fabricated from 52-μm-i.d. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) and were used throughout this study. For each, the polyimide coating was removed in a flame, and the capillary exterior was cleaned with acetone. The remaining fabrication steps were performed under a stereoscope (Leica Microsystems, Bannockburn, IL). The capillary was sectioned into 300-μm lengths using a diamond-tip fiber-optic score (Delaware Diamond Knives, Wilmington, DE). The side of each section was affixed to a piece of a microscope slide with epoxy. The section was positioned so that half of it was extended over the edge of the microscope slide. An electrochemically chloridized silver wire (50-μm diameter) (Goodfellow Corp., Berwyn, PA) was inserted into the capillary and sealed with Ag/AgCl epoxy (Metech Inc., Elverson, PA), leaving a void volume that had a length of ∼50−100 μm (Figure 1, inset). The void space determines the useable volume of the vial and can be varied by varying the insertion depth of the silver wire. Actual microvial volumes were calculated from the inner diameter and length of the void. Connection to the chloridized wire was made with a silver wire attached with Ag epoxy (Epoxy Technology, Billerica, MA), allowing for connection to the potentiostat. The exposed wires on the surface of the microscope slide were insulated with epoxy.

In some vials, the walls were coated with the silanes Polybrene or polyethylimine, as previously reported in fused-silica capillaries[16] or with the polycation poly-D-lysine. Solutions of the silanes were injected into a vial for the times described,[16] and the vials were rinsed thoroughly with both water and buffer solution before use in experiments.

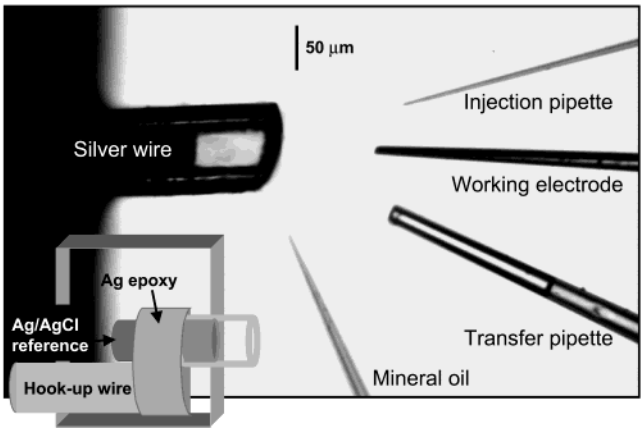

Figure 1. Photograph of the experimental components for electrochemical studies of a single cell. The vial (left side) is placed horizontally, allowing for manipulation of each pipet or electrode into the open end of the vial. The reference electrode is an integral silver wire within the vial, and the working electrode is a carbon fiber microelectrode (3 μm radius). Analyte delivery was achieved by pneumatic pressure ejection from the pulled glass capillary; cell delivery was performed using a hydraulic microinjector. A mineral oil cap was placed on the vial to prevent solution evaporation. Inset: Illustration of the completed microvial with integral reference electrode.

Electrochemistry. The integrated Ag/AgCl wire served as the reference electrode in each vial. Disk-shaped carbon fiber microelectrodes were prepared as described previously from 3-μm-radius carbon fibers (Amoco Performance Products, Greenville, SC).[17] A carbon tip was exposed by polishing on a polishing wheel (Sutter Instrument Co., Novato, CA). Electrodes were backfilled with a solution of 4 M potassium acetate and 150 mM KCl.

Electrochemical measurements were performed with the reference electrode serving as the counter electrode. For amperometry, the applied potential was +650 mV, and current was measured with a picoammeter (SR-570, Stanford Research Systems, Sunnyvale, CA). Voltammetric data were collected using locally written Labview software (National Instruments Corp., Austin, TX) with an EI-400 potentiostat (Cypress Systems, Lawrence, KS).[18] The applied potential was scanned at 300 V/s from −400 to +1000 mV or from −500 to +1500 mV, as noted. Voltammograms were repeated at 0.1-s intervals, and the results shown have been background-subtracted.[18] Electrodes were calibrated in a flow injection analysis system[19] with 10 μM dopamine before use.

Apparatus. Experiments with the microvials were performed on the stage of an upright microscope (Fisher Scientific Inc., Suwanee, GA) equipped with long working distance objectives on a vibration-free platform inside a Faraday cage. The vial was placed in a horizontal orientation beneath the objective for the experiments. Five manipulators (four MN-153, Narishige Instruments, East Meadow, NY, and one 462 Series, Newport Corp., Irvine, CA) were used for precise positioning of the vial, electrode, and injectors beneath the objective. Microvials were cleaned

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between experiments by several rinses with experimental buffer and water.

Delivery of cells and buffer solution into the vials was accomplished using cell transfer pipets (Eppendorf TransferTips ES, Brinkmann Instruments, Inc., Westbury, NY) connected to a hydraulic microinjector syringe, thus allowing for precise control of solution dispensing (IM-26, Narishige). The tips of the transfer pipets were bent 30° and had an internal tip diameter of 15 μm, allowing for monitoring of the cell during transfer from the culture plate into the vial.

Injectors for delivery of compounds into the vial were prepared by pulling glass capillaries (1.2 mm × 0.68 mm, A-M Systems, Everett, WA) with internal tip openings of 2-μm diameter in a horizontal, programmable micropipet puller (PC-87, Sutter Instrument Co., Novato, CA). A five-step program was used to optimize the shape and size of the pipet tip so that fabrication was reproducible and cutting or fire polishing of the tips was unnecessary. Injections were performed using a pneumatic microinjector (Picospitzer II, General Valve Corp., Fairfield, NJ). The injected volumes were calibrated in the vial for each injection pressure or time by measurement of the displacement of the mineral oil cap used to prevent solution evaporation.

Single Cell Manipulation. To place a cell inside the vial, the vial was submersed into a cell culture plate containing the cells of interest. The vial was filled with buffer with the transfer pipet because surface tension prevented filling of the vial upon submersion. A single cell (15 μm diameter) was harvested from the plate into the transfer pipet with a small, negative fluid displacement. The transfer pipet was then translated into the vial, and the cell was ejected by a small, positive fluid displacement. Once in the vial, the cell was allowed to rest for approximately 10 min. Then the cell culture plate was removed by lowering the microscope stage, a carbon fiber microelectrode was manipulated into the vial, and the vial was capped with mineral oil. Dopamine was then injected into the vial for uptake studies. Experiments were terminated 20 min after initial insertion to ensure cell viability. Only those cells with intact membranes, as verified visually, were used. The viability of some cells was monitored with the stain Trypan Blue during manipulation.

Cell Culture. HEK-293 cells were used that had been stably transfected with the dopamine transporter.[20] Cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 5% CO_2 and 37° C with 200 μg/mL Geneticin added to the transfected cells. Cells were rinsed twice with buffer and treated with 10 mM EDTA to promote detachment from the cell plate prior to manipulation into the vial for the transport experiments. They remained viable under the mineral oil cap, consistent with prior studies.[21]

Reagents. All solutions were prepared in doubly distilled water (Corning Mega-Pure MP-3A, Corning, NY). All chemicals were obtained from Sigma (St. Louis, MO) and were used as received. The buffer solution for experiments contained (mM): 150 NaCl, 5 KCl, 10 Tris-HCl, 2 $CaCl_2$, 1.2 $MgCl_2$, and 5 glucose at pH 7.4 with NaOH. The concentrations of dopamine in the pipet for injection into the vials were either 50 or 100 μM, and the ruthenium(II)-tris-bipyridine for the internal standard was 100 μM.

RESULTS AND DISCUSSION

Microvial Characterization. The picoliter vials fabricated from capillary tubing are transparent and have a well-defined volume. Both features are useful for manipulation of small volumes and single cells. Experiments within these vials require individual manipulation of several pipets on the stage of a microscope (Figure 1). As shown, the vial is placed horizontally under the microscope objective with each pipet accessing the vial interior through the open end. Solution evaporation of these small volumes (ranging from 100 to 200 pL in this work) is extremely rapid, occurring within 5 s after removal of the delivery pipet and exposure to the laboratory environment. The evaporation leads to an increase in solution osmolarity when the delivery pipet remains in the vial since the water evaporates and the vial is continually refilled with NaCl-containing buffer. In addition to increasing the effective working volume of the vial, this effect would be detrimental to the viability of a cell within the vial. Although several methods to avoid evaporation have been proposed,[8] we chose a mineral oil cap. By quickly capping the vial with mineral oil after filling, experimentation for several minutes was allowed without significant evaporation.

Analyte injections were performed by rapid insertion of the injection pipet into the vial, followed by pneumatic delivery and withdrawal. This prevented analyte leakage into the vial. Insertion of the injection pipet resulted in a small capacitive increase detected at the carbon fiber microelectrode during FSCV due to the increased solution volume within the vial. Calibration of the injected volumes was performed by measuring the displacement of the mineral oil/buffer interface at varying injection pressures and times using reproducibly fabricated injection pipets.

Fast-scan cyclic voltammetry was employed to monitor the concentration of electroactive species in the vial. Upon pressure ejection of dopamine, the cyclic voltammetric current rapidly increased (Figure 2) indicating rapid delivery and distribution of the electroactive species throughout the vial. For a vial of 100 pL, the interior surface area-to-volume ratio is 77 m^2/L. The magnitude of this ratio and the negatively charged surface of fused silica raised concerns of adsorptive loss of dopamine to the vial walls. However, the observed currents arising from cyclic voltammetry in vials coated with positively charged silanes prior to experimentation did not differ in the time to reach a uniform concentration or in amplitude when compared to untreated vials (data not shown). These results indicate that, similarly to the absence of significant protein adsorption to the walls of untreated fused-silica capillaries,[16] dopamine loss to the walls does not occur to an appreciable extent. Therefore, during subsequent experiments, the vials were used without pretreatment of the inner walls. For both silane-coated and uncoated vials, the oxidative current for dopamine decreased slightly with time, presumably as a result of the oxidation of dopamine by ambient oxygen.

Different volumes of a dopamine solution (50 μM) were introduced into the microvials by pneumatic injection for 5−30 ms at 10 psi. The actual volume was estimated by measurement of the displacement of the meniscus using the eyepiece reticle as a scale (1.5-μm resolution). The displacements varied from 3 to 15 μm. The calculated final concentration was plotted against the

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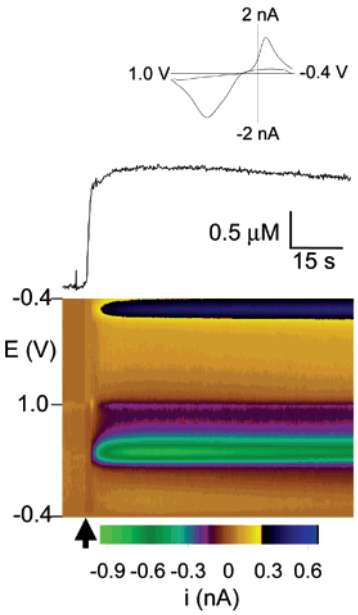

Figure 2. Cyclic voltammetry monitoring of dopamine injected into a 150-pL vial. Dopamine (50 μM) was injected (10 psi, 10 ms) into the vial in the absence of a cell at the time indicated by the arrow. The upper trace is a single cyclic voltammogram recorded while dopamine was present in the vial. The middle trace shows the current versus time for the dopamine oxidative peak occurring at +0.6 V. The amplitude indicates a final concentration of 1.5 μM based on subsequent calibration. The color plot gives the oxidative and reductive current (shown in false color) as a function of potential during the voltammetric scan and time of the scan. The signal for dopamine remains unchanged once injected. Scan rate: 300 V/s, 10 Hz repetition rate.

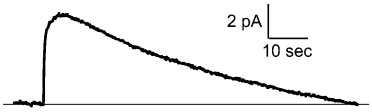

Figure 3. Amperometric monitoring of dopamine injected into a 150-pL vial. Current was monitored at +0.650 V vs Ag/AgCl with a 3-μm-radius electrode, and the injection duration was 10 ms at 10 psi. Following injection of 550 amol of dopamine, the signal increased and then slowly returned to baseline, indicating electrolytic consumption of dopamine.

dopamine concentration obtained from the cyclic voltammetry peak current in the microvial. The peak current was converted to concentration on the basis of the precalibration of the electrode in a flow injection analysis system. A linear relationship (R^2 = 0.92) was found with a slope of 0.98. Thus, quantitative calibration of vial concentrations is possible based on the displacement distance despite the fact that the meniscus is curved.

Amperometric Sampling. Amperometry was employed to determine the consumption rate and sampling efficiency of the electrode in response to injections of dopamine into the vial. Using a 3-μm carbon-fiber electrode that has a surface area of 3×10^{-11} m^2, current generated by oxidation of injected dopamine returned to baseline within 1 min (Figure 3b). For the trace shown, integration of the current versus time led to a value of 480 amol oxidized at the electrode surface that indicates almost complete electrolysis over this time scale of the 550 amol injected. Previous experiments using slow-scan cyclic voltammetry demonstrated a similar behavior for vials of this volume.[8,22]

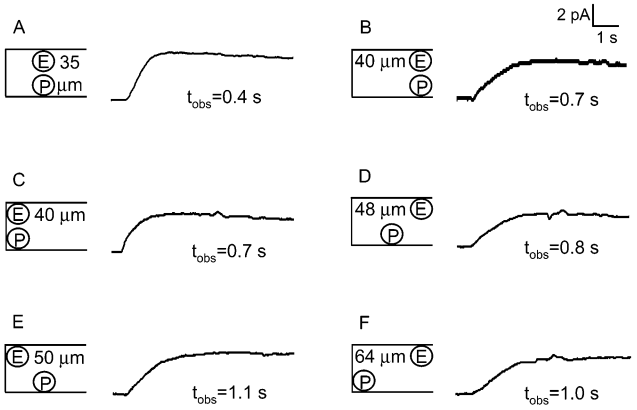

Figure 4. Mixing times in the vials as a function of electrode and injection pipet position (injection duration was 10 ms at 10 psi, giving a volume of ~10 pL) measured with amperometry. To the left of each trace, the positions of the electrode (E) and injection pipet (P) are schematically shown relative to the base of the vial. The distance between their tips is given in micrometers, and t_{obs} is the time for the signal to reach half-maximal value. The calculated times for diffusional mixing were A, 0.6; B,C, 0.8; D, 1.2; E, 1.3; and F, 2.0 s.

The pneumatic injection of dopamine into the vial causes convection that quickly mixes the vial contents, leading to a uniform analyte concentration. Even in the absence of convection, the small dimensions of the vial would provide rapid diffusional broadening based on $l = (Dt)^{1/2}$, where l is the distance for a molecule to travel, D is the diffusion coefficient, and t is the time for diffusion. For example, with the electrode sensing surface placed 40 μm from the injector tip, the meantime to cross this region would be 0.4 s. The observed mixing time was found to depend on the relative location of the electrode and injection pipet (Figure 4). With close proximity, a rapid rise time is observed, whereas when the electrode and injection pipet are further apart, the observed mixing times decrease. In all cases, convection caused by the injection plays an important role, because the mixing times are more rapid than those calculated on the basis of diffusion. These experiments were also used to determine optimal locations for pipet and electrode placement within the vial. It was found that when the electrode and injection pipet were placed in the center of the vial, mixing times were minimized, as was the current spike from the transient flow of the concentrated bolus of analyte past the electrode surface.

Cellular Uptake. The rapidity, stability, and quantitative nature of dopamine delivery with the methods described allow for the monitoring of uptake kinetics at a single cell. The transport kinetics of dopamine into transfected HEK-293 cells have been previously characterized from populations of cells using rotating disk electrode techniques.[15,20] The observed transport was blocked in the presence of cocaine, indicating specific uptake of dopamine into the cells. Additionally, transport of dopamine into nontransfected HEK-293 cells was not significant.[23] Traditionally, transport has been studied using radiolabeled compounds, which do not allow for monitoring of the kinetics in real time.

Continuous electrochemical monitoring of the vial contents during the transport process allows for the rate of dopamine uptake into a single HEK-293 cell to be measured from its rate of

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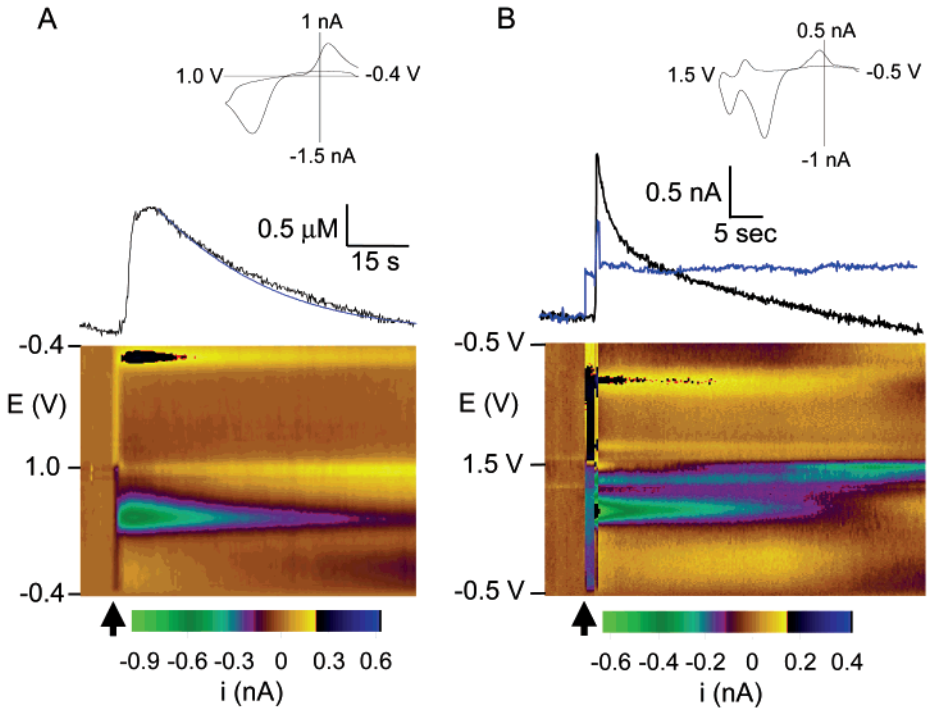

Figure 5. Cyclic voltammetric monitoring of chemical changes occurring in a picoliter-volume vial containing a single cell, transfected with dopamine transporter. In both panels, the upper trace is a single background-subracted cyclic voltammogram recorded at the maximum response, the middle traces are the current at potentials selected for specific substances injected (converted to the concentration in A), and the color plot represents the oxidation and reduction currents as a function of applied voltage and time. (A) Responses recorded following injection of dopamine that was injected at the time indicated by the arrow. Superimposed on the middle plot is a curve calculated for Michaelis−Menten kinetics. Applied potentials and scan rates are the same as in Figure 2. (B) Responses recorded following the injection of a mixture of dopamine and ruthenium(II)-tris-bipyridine, which served as an internal standard. The potential was scanned from −0.5 to +1.5 V and back at 300 V/s at a 10 Hz repetition rate. The internal standard has a peak potential of 1.35 V vs Ag/AgCl. The middle trace shows that the current for oxidation of ruthenium(II)-tris-bipyridine (blue) remains constant, but that for dopamine (black) decreases as a result of uptake into the cell. This is also seen in the color plot. The initial rise in current is due to capacitance from insertion of the injection pipet into the vial.

disappearance from the extracellular fluid. These results can be used to determine the initial transport rate, V_{max}, on the basis of the Michaelis−Menten model of saturable kinetics. The slope of the curve immediately following injection of dopamine was used along with the K_m value, which is 1.6 μM for these cells,[23] to calculate V_{max}. In the example shown in Figure 5a, the initial vial concentration was 1.5 μM, and within 60 s, it had been cleared from the vial. From curves such as these from 12 cells, the mean value for V_{max} was 370 ± 110 nM/s. Multiplying this value by the vial volume gives a V_{max} of 55 ± 17 amol/s/cell. When measured in populations of cells (~10^6 cells) a value of 18.9 ± 1.4 amol/s/cell was reported.[23]

The difference in these values could be due to a sampling bias in the single cell measurements so that the heterogeneity in transporter expression among individual cells was not averaged away. Alternatively, the difference could be due to the larger cell volume/solution volume ratio used with the single cell experiments. The transport rates from populations of cells were measured using the rotating disk electrode with one million cells in 300 μL. The single-cell measurements involve a cell of ~2 pL volume in a volume of 100 pL. Thus, in the vial experiment, the cell volume/solution volume ratio is 0.02; however, it is 0.006 for the rotating disk electrode experiments. The lower extracellular volume per cell in the vial experiments means that the total number of dopamine molecules transported into each cell will be lower. Thus, the inner concentration will not reach so large a value and reverse transport, a documented phenomena in these cells,[20] will be less likely to occur. Consistently with reverse or outward transport, in some cells examined, the dopamine concentration did not go to 0, but after an initial period of uptake, a plateau of dopamine concentration was reached. If these had been included in the average, it would have lowered the V_{max}. In neurons, cytoplasmic accumulation of dopamine is less likely to occur because of intracellular metabolism or packaging into vesicles.

Addition of an internal standard allowed the solution volume and electrochemical response during uptake experiments at cells to be monitored simultaneously. Ruthenium(II)-tris-bipyridine was used, since it is soluble in aqueous solutions and can be detected electrochemically (E_p = 1.35 V vs Ag/AgCl). This compound cannot be transported across the cell membrane and has been shown not to have deleterious effects on cell function or membrane viability at concentrations lower than 1 mM.[24] In experiments using this internal standard, its oxidative current remained constant over the time course of the experiment, whereas that of dopamine decreased in the presence of a single cell (Figure 5b). This indicated that no significant variations in solution volume or electrode response were taking place during the time course of the experiment and that the decrease in dopamine concentration observed is due to uptake by the cell.

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This novel approach to microvials allows for the characterization of the chemical dynamics of electrochemically active species in picoliter volumes. The measurement of the analyte introduced into vials using electrochemical techniques shown here provides a system useful for the study of low concentrations of molecules without the disadvantages of dilution or diffusional dispersion. The results obtained in these vials exhibit reasonable volumetric accuracy and precision. Furthermore, the preliminary results at the single cell level indicate feasibility for quantitative uptake studies. Therefore, using this approach in confined volumes, quantitative single-cell physiology to learn about their individual properties that are not apparent in populations of cells should be possible. The main disadvantage of this approach is the difficulty of manipulating single cells in confined volumes while maintaining their viability.

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